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UNIVERSITY OF MISSOURI COLLEGE OF AGRICULTURE
AGRICULTURAL EXPERIMENT STATION

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Virus Protein of Mosaic Disease of Tobacco

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SUMMARY

Anhydrous solid sodium sulfate may be used to salt out the virus from the juice of diseased plants or from more highly purified fractions.

The virus readily separates in the crystalline-like form of Stanley when anhydrous solid sodium sulfate is added to the virus fraction after the reaction has been adjusted to a hydrogen ion concentration equal to pH 4.0.

Most of the ash may be removed from the crystalline-like material by washing with dilute acetic acid. The nitrogen content is about sixteen per cent, and phosphorus is present.

All visible traces of the brown pigment normal to juice from tobacco plants may be removed from the purified fraction by shaking a phosphate dispersion with ether. The pigment is carried to the top as the droplets of ether rise. The ether and brown pigment may then be easily drawn off.

X-ray diffraction patterns of tobacco mosaic virus protein prepared by the sodium sulfate method were obtained. These patterns do not agree very well with those obtained by Wyckoff and Corey who used virus protein prepared by the ammonium sulfate method of Stanley.

There was no indication on any film of spacings greater than 14.3A, although spacings as great as about 50A could have been detected. Wyckoff and Corey recorded six spacings greater than 14.3A.

Spacings as low as 2.43A were obtained, in contrast with the smallest spacing of 3.39A recorded by Wyckoff and Corey.

The absence of long spacings in virus protein prepared by the sodium sulfate method may indicate that it is composed of smaller molecules, and hence has a lower molecular weight than that studied by others.

Virus Protein of Mosaic Disease of Tobacco*

C. G. Vinson, D. K. McReynolds, and N. S. Gingrich

In 1937, in studying the virus protein of mosaic disease of tobacco, a means was found of obtaining consistent results in taking the virus fraction out of solution in crystalline-like form, without employing a nitrogenous salt to secure this result. Solid anhydrous sodium sulfate may be successfully used to salt out the virus fraction from juice of diseased plants or from more highly purified virus fractions. Acid may be used, but is not required. The amount of sodium sulfate required varies with the preparation—virus and hydrogen ion concentration being determining factors. In the case of untreated juice from diseased plants, an amount of solid anhydrous sodium sulfate sufficient to half saturate a like volume of water is usually ample to salt out most of the virus. In the case of more highly purified fractions, only a very slight amount may be necessary to cause the virus to separate in the characteristic crystalline-like form, when the concentration of virus is one to two

Fig. 1.—Crystalline-like material (x 1200) from juice of Turkish tobacco plants diseased with Johnson's virus No. 1. (8).

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per cent (in M/15 phosphate) and when the hydrogen ion concentration is equal to about pH 3.5 to 4.5. The crystalline-like material (Fig. 1) may be redispersed and recrystallized apparently any number of times. The product may be practically freed of ash by washing with dilute acetic acid of pH 3.5. The ash content of such a product is very low indeed (see Table 1). Ross and Vinson (11)* have already reported virus fractions with an ash content of less than one per cent. The nitrogen content is about 16 per cent. Phosphorus is present.

TABLE 1.—ASH AND NITROGEN CONTENT OF THE WASHED CRYSTALLINE PRODUCT

Sample	Per cent Ash	Per cent Nitrogen
1	.55	15.8
2	1.52	16.1
3	.32	16.2
4	.58	16.0
5	.80	15.9

The last visible traces of the brown pigment normal to juice from tobacco plants is readily removed by vigorously shaking a neutral phosphate dispersion of the crystalline-like product with a volume of ether about thirty per cent that of the suspension. The ether emulsifies in the water, and as the ether and aqueous phases separate, the pigment is carried along on the films of the droplets of ether in the water. Separation of the ether phase may be hastened by contrifuging. The aqueous phase may then be drawn off, or the ether, along with the suspended pigment, may be poured off after separation of the phases has been aided by centrifuging. Two such treatments with ether are usually quite sufficient to free the virus fraction from the pigment. The crystalline-like product after such treatment gives a uniform white product when packed in a centrifuge tube by centrifugation.

The activity of this product, parallels that obtained by the ammonium sulfate method (13). The crystalline-like bodies are on the average 30-40 microns long and about one micron across. Much larger particles may be obtained by salting out the virus from more concentrated solutions—one to two per cent. One to two per cent virus fractions dispersed in M/3 phosphate solution with a hydrogen ion concentration equal to about pH 7 have been observed to separate out in very long crystalline-like bodies when allowed to stand several days at room temperature. The width of such bodies does not increase in proportion to their length. Such fractions in

*Numerals in Parenthesis refer to "Literature Cited", Page 12.

tubes have a distinct pearly lustre. At first these bodies are readily redispersed by shaking; but on longer standing the process is not readily reversed by this treatment. These observations are in accordance with those of others (3).

The virus may be salted out in crystalline-like form from any of the virus fractions purified by the various methods already described. To make the initial precipitation of the virus from juice of diseased plants, the lead procedure (15), the safranin procedure (16), or the acid procedure (18) may be used, or solid anhydrous sodium sulfate may be used, with or without acid. In case the virus is salted out from the juice in the first place, it is preferable to add acid to the juice until the hydrogen ion concentration is equal to a pH of about 4.0. Much less sodium sulfate will then be required to complete the precipitation. When sodium sulfate alone is employed to salt out the virus from the original juice or from any dilute solution, so much of the salt may be required that the virus fraction will rise to the top of the liquid instead of settling, due to the increased density of the medium. The safranin method yields a good product without much manipulation. The most direct procedure, however, is to make the initial precipitation with a small amount of solid anhydrous sodium sulfate following acidification of the juice to a hydrogen ion concentration equal to about pH 4.0.

Dilute phosphate solution with a hydrogen ion concentration equal to about pH 7 continues to be the most effective dispersing medium. Very dilute sodium carbonate or potassium hydroxide give more opalescent dispersions. More concentrated solutions in dilute potassium hydroxide are quite viscous and distinctly less satisfactory to handle. It might be expected that a solution of potassium thiocyanate would peptize the virus fraction more effectively than phosphate. This is not true of those solutions used so far (N/1 with a hydrogen ion concentration equal to about pH 7). The same may be said of N/1 potassium iodide solution of the same reaction.

A fifteenth molar solution of sodium citrate is a fairly satisfactory medium in which to disperse the virus (6). An M/15 sodium citrate solution has a hydrogen ion concentration equal to about pH 7.0. Sodium citrate solution seems to be more effective when the virus is first dispersed in distilled water, then the proper amount of M/3 sodium citrate solution added to make the final concentration about M/15. Such a procedure is not necessary to obtain good dispersion with dilute phosphate solution. Solid aluminum sulfate

[$Al_2(SO_4)_3.18H_2O$] or solid anhydrous sodium sulfate may be used to salt out the virus, in the characteristic crystalline-like form of Stanley, when sodium citrate solution is the dispersion medium. Obviously aluminum sulfate cannot be used when phosphate solution is the dispersion medium.

DISCUSSION

An active crystalline virus fraction which retained activity on recrystallization was described by Vinson and Petre before Section C at the New York (1928) meeting of the American Association for the Advancement of Science. The fraction was relatively high in ash, and subsequent attempts to consistently obtain crystals having very little or no ash by means of acetone were unsuccessful. At the American Association for the Advancement of Science meeting in Cleveland (1930), Vinson presented a paper before Section C on "Isolation of an Active Virus Fraction Very Low in Ash". These fractions were obtained by careful acidification. Some of the fractions obtained at that time in the manner described contained the characterstic crystalline-like particles, which were similar to those more recently described by Stanley (12) and obtained from acid solution by using ammonium sulfate to force the virus out of solution. At that time (1930) the results were not consistent. Sometimes the virus fraction would separate as a crystalline-like product; but other times it did not. Acetone could not be relied upon to cause the virus to separate in this particular form.

Vinson and Petre (14) demonstrated in 1929 that ammonium sulfate could be used to salt out the virus from juice of diseased plants. The use of ammonium sulfate was never seriously considered since this salt is nitrogenous and because aluminum sulfate solution was soon found superior (17). It is obvious that the use of a nitrogenous salt to salt out a nitrogenous body is not desirable. The data in the literature fully support this statement. The crystalline virus fraction obtained by means of ammonium sulfate was reported in 1935 (12) to have a nitrogen content of twenty per cent. More recently it is estimated to have a nitrogen content of 16.6 (13) to 16.7 (2) per cent. These data suggest that the crystalline-like virus fraction reported in 1935 consisted mainly of ammonium sulfate, since ammonium sulfate has a nitrogen content of about 21.2 per cent. Certainly the crystalline-like product obtained in 1935 by salting out with ammonium sulfate was higher in inorganic constituents than the one obtained through the use of dilute acetone and reported in 1931 (15). No work has been reported to date which discredits the

crystalline product from dilute acetone. Others (4) who have investigated the crystalline product from acetone have apparently used strong acetone (two volumes of acetone to one volume of virus phosphate solution) and also apparently worked at room temperature (4). In our experience, this procedure almost completely inactivates the virus at the first precipitation. Vinson and Petre (14) carried out their precipitations with strong acetone in a cold room where the temperature was never above —10°C. and usually about —15°C. when the precipitation was carried out. The acetone precipitate thus obtained was never suspended in phosphate solution, redistilled water only being used. The precipitate readily dispersed in redistilled water. After first clearing the juice from diseased plants with basic lead acetate solution, then precipitating two or three times in the cold with strong acetone (two to one), the virus fraction was taken up in redistilled water, centrifuged and the more or less clear supernatant liquid was carefully concentrated to a small volume in vacuo. This concentrated solution, as indicated in 1931 (15), was brought to a hydrogen ion concentration equal to about pH 5.0 with acetic acid. Acetone was then added slowly with constant stirring until a slight permanent cloud appeared. The final acetone concentration was always less than thirty per cent of the total volume, for acetone concentrations above forty per cent markedly inactivate the virus at ordinary temperatures (1). This was the primary reason for concentrating in order to reduce the concentration of acetone required. No suggestion was made (15) that the crystalline product from dilute acetone represented pure virus.

X-RAY DIFFRACTION STUDIES OF TOBACCO MOSAIC VIRUS PROTEIN PREPARED BY THE SODIUM SULFATE METHOD

It is well known that x-ray diffraction studies of tobacco mosaic virus protein have shown that this material exhibits a crystalline diffraction pattern. Furthermore, it has been reported (2) that x-ray diffraction patterns produced by different fractions of virus material are not identical. It is not unreasonable, therefore, to expect differences in the diffraction patterns for this virus material when prepared by different methods. In the present work, x-ray diffraction patterns of tobacco mosaic virus protein prepared by the sodium sulfate method have been obtained, and they are compared with patterns obtained by previous investigators using the same type of virus material prepared by other methods (12).

In order to study the x-ray diffraction pattern of virus materials, a camera having certain special features is required. Two special

features are, first, that the sample-to-film distance should be large, and, second, that the camera should be air tight. Figure 2 shows the essential details of the camera used in this work. The collimating tube T is provided with removable end pieces having circular holes of several diameters ranging from 0.5 mm. to 1.5 mm. The outer collimating hole was covered with thin nickel foil for the double purpose of making the camera gas tight and for x-ray filtering. The sample holder S, when prepared, was placed in the hole H and clamped against a gasket with three wing nuts. The film holder F was clamped against a gasket on the back of the

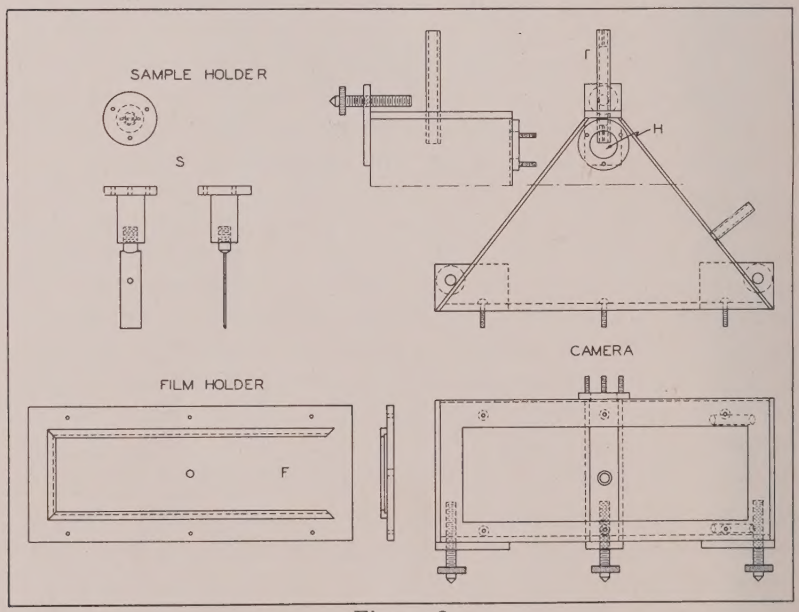

Figure 2.

camera. Levelling screws and a small central hole in the film holder facilitated lining up the camera. A small funnel-shaped piece of brass was used to prevent the direct beam from striking the film. An inlet tube and an outlet tube were used to pass helium gas through the camera.

The sample-to-film distance was determined by direct measurement and also by calibration with the diffraction pattern of an aluminum wire. The perpendicular distance from sample to film was found to be 15.41 cm.

The virus material, a pasty mass, was placed in the hole of the sample holder, and sealed with faces of thin-blown glass about 0.001 cm. in thickness. Agfa double emulsion, non-screen film, covered with thin black paper, was placed in the film holder which was then clamped to the camera. Radiation from a copper target tube passed through the filter, the collimating holes and the sample, and the diffracted beams were registered on the photographic film. Exposures of the order of 100 hours were required to obtain measurable lines. Helium gas in the camera reduced the general background scattering and reduced the time of exposure appreciably.

Fifteen diffraction patterns were obtained with samples from several batches of virus material. Several attempts were made to align the crystals by means of flow orientation and by means of electric currents, but the best patterns were obtained with unoriented material. Six of the patterns were sufficiently clear and intense for making reliable measurements. They were not all identical—certain very weak lines appeared on some films but not on others. If the evidence for the existence of such weak lines appeared doubtful, they were omitted. In a few cases, where the general patterns were in good agreement, a few lines on each pattern appeared to be shifted slightly from the average positions as obtained from the other films. The positions of the diffraction lines on the four films which showed the greatest consistency were averaged and from these average positions, the angles of diffraction were obtained. From these angles and the wavelength of the x-rays, the interplanar spacings were calculated from Bragg's law. The spacings so obtained, and the relative intensities of the diffraction for each spacing, are given in Table 2 and Figure 3. In Figure 3, the upper lines represent spacings and intensities obtained in the present work while the lower lines represent those obtained in the work of Wyckoff and Corey. Comparison of this data is made with that of Wyckoff and Corey (19) who used virus material prepared by Stanley. In Table 2, an estimate of the maximum error of measurement is given.

There are several striking differences between the patterns obtained by Wyckoff and Corey using Stanley's material, and those obtained in the present work. First, there was no indication on any film of spacings greater than 14.3A, although spacings as great as about 50A could have been detected. This is to be contrasted with the fact that six spacings greater than 14.3A were recorded by Wyckoff and Corey. Second, spacings as low as 2.43A

have been obtained, in contrast with the smallest spacing of 3.39A recorded by Wyckoff and Corey. Third, nearly half of the spacings here obtained are in poor agreement with those listed by Wyckoff and Corey. Fourth, the relative intensities in the two cases are generally different.

TABLE 2.

Experimental		Wyckoff and Corey	
I	d	d	I
VW	2.43±0.01	3.39	W
W	2.82±0.02	3.54	VW
W	2.90±0.02	3.70	VW
W	3.21±0.02	3.88	M
M	3.49±0.03	4.08	W
M	3.71±0.03	4.44	M
MW	3.94±0.03	4.71	M
W	4.13±0.04	4.95	M
M	4.36±0.04	5.44	W
W	4.62±0.04	5.7	VW
VW	5.06±0.05	6.5	W
M	5.37±0.06	7.44	M
VW	5.79±0.07	9.2	M
VS	7.0±0.1	10.2	W
W	7.7±0.1	11.0	S
W	9.1±0.2	14.2	VW
S	11.1±0.3	16.2	VW
VS	14.3±0.4	20.8	M
		28	VW
		37	W
		53	M
		80	S

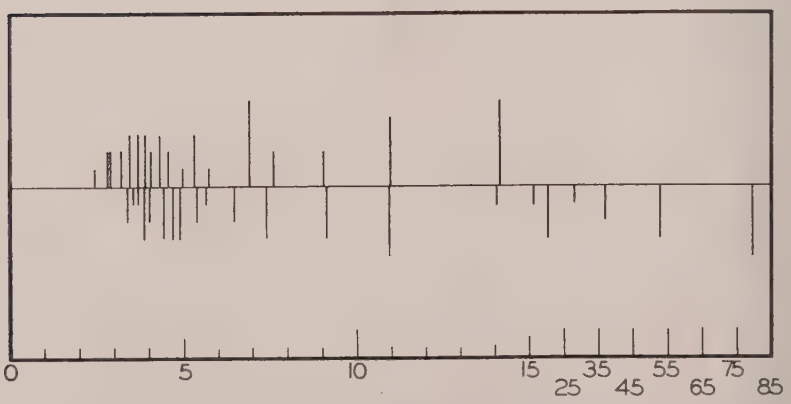

Figure 3.

Assuming the absence of impurities in the samples here used, and those used by Wyckoff and Corey, and assuming the patterns arise from interferences within the molecule, it must be concluded that there is a difference in the structures of the virus molecules of the two materials. The presence of inorganic impurities is unlikely in either material. The presence of organic impurities is, perhaps, not so definitely ruled out. The work of Bawden, Pirie, Bernal and Fankuchen (2) indicates that the spacings here obtained are intramolecular spacings rather than intermolecular spacings. They conclude, also, that the physical properties of crystalline virus materials are best explained by postulating rod-shaped molecules of estimated length of at least 1000A.

The absence of long spacings in the present material may indicate that it is composed of smaller molecules, and hence has a lower molecular weight than that studied by others. From estimations of dimensions of the virus particles, Hills and Vinson (7) calculate the molecular weight of their material as about $2x10^6$. Eriksonn-Quensel and Svedberg (5) have estimated the molecular weight of their virus materials as about $17x10^6$. More recently, the molecular weight of certain virus material has been given as high as $63.2x10^6$ (9) and $64.8x10^6$ (10). Thus, the present study is in at least qualitative agreement with other work in indicating the probability of the existence of smaller molecules in the material obtained by the sodium sulfate method.

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